

The University of Chicago

CHEMICAL FACTORS IN NERVE DEVELOPMENT

**A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE BIOLOGICAL
SCIENCES IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**

**DEPARTMENT OF ZOÖLOGY
SEPTEMBER, 1943**

**By
AGNES SANXAY BURT**

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CHEMICAL FACTORS IN NERVE GROWTH STUDIED IN TISSUE CULTURE: VITAMIN B₁ AND THE GROWTH OF SPINAL GANGLIA

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ONE FIGURE

INTRODUCTION

In spite of widespread clinical use of vitamin B₁ (thiamin hydrochloride) in a variety of pathological conditions of the nervous system, the exact mode of action of this substance upon nervous tissue has not been determined. It is known only that vitamin B₁ is necessary for the growth of young animals, for the maintenance of the nervous system, and for the regeneration of severed nerves.

In 1939 Banga, Ochoa, and Peters showed that phosphorylated thiamin is identical with cocarboxylase, which catalyzes the decarboxylation of pyruvic acid in the organism. There is also considerable evidence that thiamin may be involved in the acetylcholine-cholinesterase system (see Ward and Kennard, '42, for a review of the literature). Thirdly, it has been reported that thiamin has a direct stimulating effect on growth. It was the purpose of the work presented in the following paper to test this last hypothesis by studying the action of vitamin B₁ on the spinal ganglia of embryonic chicks in tissue culture.

Effects of vitamin B₁ deficiency on the nervous system

Most of the literature on B₁ and the nervous system deals with the effects produced by vitamin deficiency. Clark in 1914 reported segmentation of axis cylinders and myelin degeneration in chickens subsisting on polished rice, which degenerative changes could be reversed upon return to a normal diet. Subsequent work with crystalline thiamin has, for the most part, confirmed the findings of Clark.

¹ This research was done under the direction of Dr. Paul Weiss in partial fulfillment of the requirements for the degree of Doctor of Philosophy. It was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago, and was aided by grants from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

Long continued subminimal intake of B_1 in the dog produces myelin degeneration of both peripheral nerves and the posterior columns of the spinal cord (Street and coworkers, '41). After prolonged deficiency the nervous system can no longer be restored to normal by the administration of thiamin. In pigeons, Swank ('40) finds that the first symptom of acute thiamin deficiency is opisthotonus, sometimes accompanied by structural changes in the nervous system consisting, depending on the duration and severity of the deficiency, of axis cylinder degeneration, myelin degeneration, and eventually of chromatolysis of the perikarya. If thiamin is administered in time, all these changes can be reversed.

In the rat (Prickett, Salmon, and Schrader, '39), while sudden acute B_1 deficiency may produce few changes in the peripheral nerves, a chronic deficiency produces edema, bulbous enlargements of the nerve fibers, and myelin degeneration.

In human beri-beri, among the first symptoms are sensory and motor disturbances in the arms and legs. Vacuolar degeneration of the Schwann cells occurs, followed by axis cylinder fragmentation and wallerian degeneration (Eddy and Dalldorf, '41). Aring and associates ('41) have found reduced myelin sheaths and fragmented axis cylinders in peripheral nerves of pellegrins suffering from multiple vitamin deficiencies. Wernicke's lesions, which sometimes occur in the brain in extreme malnutrition, may be identical with the lesions produced in the brains of pigeons by prolonged B_1 deficiency (Alexander, '40).

Clinical effects of vitamin B_1

The medical literature on the effects of thiamin in pathological states is so extensive that no attempt will be made to review it all. Most of it deals with relief of neuritis in a wide variety of conditions (Spies and Aring, '38; Heiman, '37; Sliosberg, '39). Thiamin has also been reported to relieve postdiphtheric paralysis, poliomyelitis, Parkinson's disease, and progressive muscular atrophy (Reich, '38; McCormick, '39; Holmin, '39). Vitamin B complex or vitamin B_1 plus nicotinic acid have been reported to restore high-tone hearing in cases of deafness (Selfridge, '39; Veasey, '40). The most extreme clinical view of the role of B_1 in nervous disorders has been stated by Lewy ('38), who says, "It has been concluded from therapeutical experiments that a B-avitaminosis is instrumental in producing many, if not all, parenchymatous neuropathies."

However, much of the clinical work on vitamin B_1 has not been well controlled. Doyle and Merritt ('41), who were unable to arrest

muscular atrophy by the use of thiamin, point out that the natural variations in the conditions in which B_1 has been used are extreme and caution against ascribing the improvements sometimes noted after administration of B_1 to the effects of the vitamin unless adequate controls are available.

Physiological effects of excess thiamin

Addition of thiamin or cocarboxylase to an avitaminotic brain suspension *in vitro* will increase the oxygen consumption; however, addition of either compound to a suspension of normal tissue has no effect (Ochoa and Peters, '38). Similarly (Tokaji and Gerard, '39), vitamin B_1 will restore the atypical brain waves of avitaminous pigeons to normal but has no effect on the brain waves of healthy pigeons.

Thiamin may be involved in the neuro-humoral system as it is known to cause contraction of eserinizied leech muscle (Byer and Harpuder, '40) and may (Minz, '38) reinforce the action of acetylcholine on isolated mammalian intestine. However, thiamin has no effect on many frog and mammalian tissues normally sensitive to acetylcholine-like substances. Thiamin increases muscle tonus in avitaminotic rat intestine but has no effect on normal intestine (Dick and Hege, '41).

Vitamin B_1 and nerve repair

When the sciatic nerve is severed in rats (Tangari, '40), no return of sensory or motor function is observed in animals kept on a B_1 -deficient diet, while those receiving supplementary B_1 apparently recover sensation and motility in the operated legs 10 to 20 days before normal controls. However, it is not clear whether the quicker return of function in the treated rats is due to increased growth of nerve fibers or to functional facilitation.

Ward and Kennard ('42) report that in two monkeys in which cortical areas 4 and 6 had been removed, thiamin increased the rate of return of motor function. They also found that it augmented the effect of choline compounds in increasing the rate of recovery. These workers definitely ascribe their results to functional facilitation.

Vitamin B_1 and growth

In young rats (Whipple and Church, '36) administration of thiamin increases body weight by increasing the amounts of fat and water in the tissues. Addition of B_1 to the medium shortens the time between egg

laying and pupation in *Drosophila* and increases body length of larvae as compared with individuals in control cultures containing no B_1 (Schrader, '41).

In some species of bacteria, thiamin (or one or both of the two organic radicles of which it is composed) acts as an accessory growth factor increasing the rate of multiplication beyond the low basal level characteristic of cultures without thiamin (Koser and Saunders, '38; Robbins, '40). In plants, although it is well established that vitamin B_1 is necessary for root growth (Robbins and Schmidt, '38; White, '41), evidence is accumulating that high concentrations of thiamin do not increase root growth to any significant extent (Hitchcock and Zimmerman, '41; Donnelly, '41; Chadwick and Swartley, '41; Thimann, '41).

Vitamin B_1 in tissue culture

Before crystalline thiamin was available for research, Rossi ('35) reported that addition of a vitamin B preparation made from brewers' yeast inhibited growth of fibroblasts at high concentrations and stimulated growth when more dilute. Baker ('36) added 0.0053 units of Burroughs Wellcome and Co.'s B_1 to her synthetic medium for monocytes but did not publish any data analyzing the effects of B_1 on growth. Hengstmann ('39) added crystalline B_1 to cultures of embryonic chick heart, human embryonic connective tissue, and chicken leucocytes and reported an inhibition of growth by thiamin at high concentrations, and a slight stimulation between 5 and 10 mg. per cent.

The work described above deals with the effects of excess thiamin in tissue culture. McCarrison and Sankaran ('33) obtained indirect evidence of the function of B_1 in nerve growth by culturing embryonic chick spinal cord in avitaminotic plasma. They found that nerve fiber growth was very sparse and degenerated much faster than in normal plasma. However, it is not clear whether their results were due to the lack of B_1 per se or to toxic metabolites in the plasma.

In view of the unsettled state of the problem, it was decided to check the effects of thiamin on nerve growth and regeneration in tissue culture. The first part of this work is presented below.

MATERIAL AND METHODS

The experimental procedure used consisted of dissecting the lumbosacral spinal ganglia out of 9- to 16-day-old chick embryos and recording the type and amount of nerve fibre outgrowth which occurred in media containing various amounts of thiamin hydrochloride as

compared with the growth which occurred in control cultures to which no vitamin was added. A total of 233 ganglia was studied, protocols for 144 of the cultures being presented here.

(It is understood throughout that "growth" in the sense of increasing living mass may not occur under these conditions. In tissue culture the neurons put out thin pseudopodia which migrate into the surrounding medium spinning out axon fibers behind them. It is not known whether these fibers take in material from the medium and actually "grow" or whether they increase in size at the expense of the cell body. However, these structures are what is meant by the term "nerve outgrowths" in this paper.)

The ganglia were cultured in single coverslip preparations made in the following manner. A paraffin ring 1 cm. in diameter was stamped on each piece of mica with the end of a hot test tube. Inside this ring a thin plasma film was made by adding a drop of plasma and a small amount of embryo extract and sucking off the excess fluid with a micropipette. Then the ganglion to be tested was placed in the center of the film and covered with a liquid medium to which, in half the cases, crystalline thiamin hydrochloride (Lederle) had been added.

The composition of the liquid medium was as follows:

Distilled water	1000 cc.
Witte's peptone	1.00 gm.
Dextrose	4.00 gm.
NaCl	5.80 gm.
KCl	0.15 gm.
CaCl ₂	0.15 gm.
MgCl ₂ ·6 H ₂ O	0.075 gm.
NaH ₂ PO ₄	0.038 gm.
Phenol red	trace

As the medium was slightly acid, the pH was adjusted to 7.4 immediately before use by the addition of a sterile solution of 1% NaHCO₃ until the phenol red indicator was of the proper color.

Cultures were incubated 2 days, as it had been previously noted that the height of nerve outgrowth under these conditions was reached on the second day. Observations were made on the living material both the first and second days of incubation. All the cultures in any one set of controls and experimentals were kept out of the incubator the same length of time while observations were being made, so that the total incubation time was the same for all cultures of any one set.

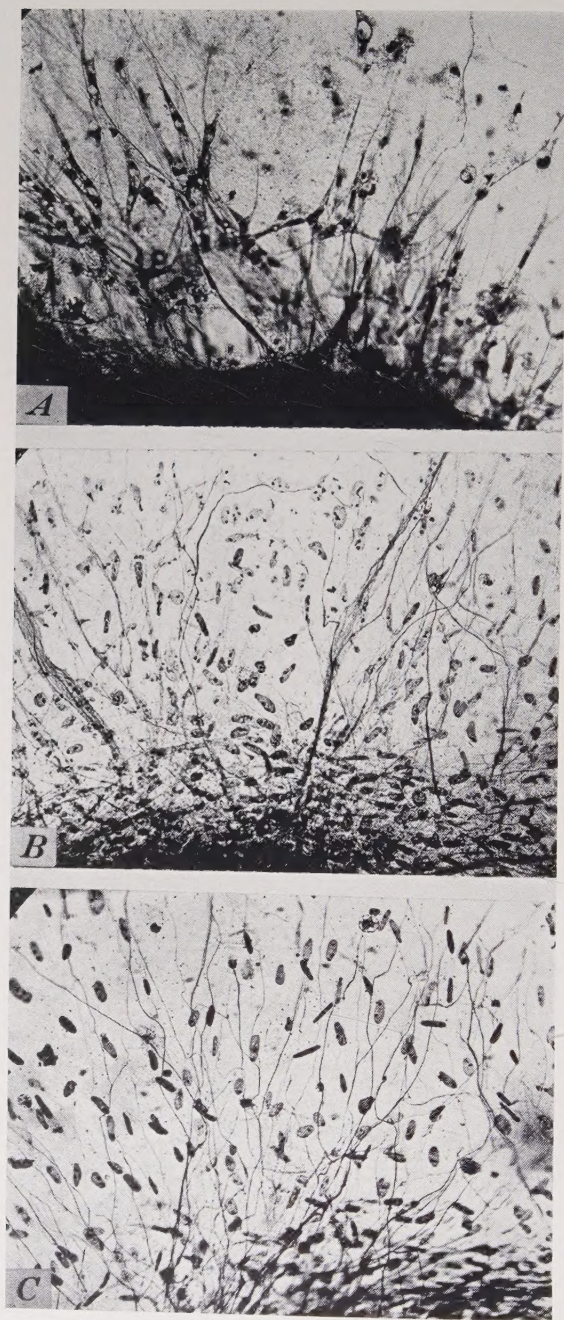


Fig. 1 A. Culture illustrating ++ nerve fiber density. $\times 230$.
B. Culture illustrating +++ nerve fiber density. $\times 230$.
C. Culture illustrating ++++ nerve fiber density. $\times 230$.

The density of fibroblasts and of macrophages was noted in the live cultures and recorded using an arbitrary system in which + signified very sparse growth, ++ moderate growth throughout the culture, +++ good growth, and ++++ very good, almost solid growth. Observations on nerve fibers in the living cultures were found to be unreliable because they were easily masked by fibroblasts. Consequently, after 2 days of incubation, each culture was fixed and stained in toto by Bodian's protargol method, the density of nerve fibers noted and their length in micrometer units recorded from the fixed and stained material. Density of nerve fibers was judged by the same arbitrary system used for macrophages and fibroblasts. Figure 1 shows cultures which conform to ++, +++, and ++++ standards of density. A grade of + for nerve fibers means that only five or six fibers emerged from the whole ganglion. As the length of nerve fibers varied considerably, the figures recorded are averages of four areas per ganglion.

RESULTS

Several concentration ranges of thiamin were tested. One group of cultures was made using B_1 in the liquid medium at concentrations of 8 to 12 mg. per cent (as the plasma film contained no added thiamin, this range corresponds to Hengstmann's cultures with 5 to 10 mg. per cent of B_1). In another group of experiments concentrations of 20 to 24 mg. per cent thiamin were tested. The results of these experiments are tabulated below.

A third group of fifty cultures was made in which concentrations of 1 to 5 mg. per cent thiamin were added to the liquid medium in half the cases. No quantitative nor qualitative differences between the behavior of ganglia in experimental and in control cultures was noted in this range. In a fourth group of thirty-nine ganglia, no liquid medium was used and small pellets² of thiamin hydrochloride were added directly to the plasma clot. Here the concentrations of B_1 were much higher and marked inhibition of growth of all types of cells resulted. This is in accord with the findings of previous workers and does not necessarily indicate a specific toxic action of the vitamin, as the pH of the cultures in these cases is lowered beyond the physiological range.

The following tables record the observations made on control cultures and on the cultures to which 8 to 24 mg. per cent thiamin were added:

² These pellets were very kindly prepared by Dr. T. F. Gallagher, of the Department of Biochemistry.

TABLE 1

Summary of experiments testing effects of 8 to 24 mg. per cent thiamin in liquid medium.

EXP. NO.	AGE CHICK	INCUBATION TIME	CLASS	NF	L	FB	MC	REMARKS
I	14 d.	39 hrs.	NC	++++	30	+++	+++	
				++++	25	++	+	
				++++	15	+++	+	
				++++	20	+	+	
				++++	25	+++	++	
				++++	25	++++	+++	
				++++	20	++	++	
				++++	25	++	+	
				++++	20	+	++	
				++	20	+	++	
				++	15	+	+	
				+	15	+	+	
			8 mg. % B ₁	++++	25	+	++	
				++++	20	++	+	
				++++	20	++	++	
				++++	15	0	0	
				++++	10	++	++	
				++++	15	+	0	
				++++	30	+++	++	
				++	5	+	0	
				++	15	+	+	
				++	10	+	+	
				++	10	+	+	
				+	15	+++	++	
II	15 d.	44 hrs.	NC	++++	20	+++	+++	
				++++	30	+++	+++	
				++++	25	++	++	
				++++	20	+++	+++	
				++++	40	++++	+++	
				++	40	++++	++++	
				++	15	+++	+++	
				++	15	++	++	
				++	35	++++	+++	Ganglion lost in staining
				—	—	++	+	
			8 mg. % B ₁	++++	40	+++	++	
				++++	40	++++	+++	
				++++	40	+++	+++	
				++++	30	+++	+++	
				++++	30	+++	+++	
				++++	25	+++	+++	
				++++	35	++	+++	
				++	15	++	++	
				++	—	++	++	2 ganglia
				—	—	++	++	Poor slide
III	14 d.	41 hrs.	NC	++++	35	+++	+++	
				++++	40	+++	++	
				++++	20	+	+++	
				++++	35	++	++	
				++++	25	+++	+++	
				++++	15	++	++	
				++++	20	+++	+++	
				++++	25	+++	+++	
				+	10	++	+++	
				+	15	++	++	
			8 mg. % B ₁	—	—	+++	+++	
				—	—	++++	+++	
				—	—	++++	+++	
				—	—	++++	+++	
				—	—	+++	+++	
				—	—	++	+++	
				—	—	++	+++	
				—	—	++	+++	Bodian stain did not take on this group of slides

TABLE 1 (Continued)

EXP. NO.	AGE CHICK	INCUBATION TIME	CLASS	NF	L	FB	MC	REMARKS
IV	15 d.	46 hrs.	NC	++++	35	++++	++++	
				++++	30	++++	++++	
				++++	30	++++	++++	
				++++	20	++	++++	
				++++	20	+++	++++	
				++	40	++++	++++	
				++	25	+	++	
				++	20	++	++	
				—	—	++	++	
			12 mg. % B ₁	++++	20	++	+	
				++++	25	+++	++++	
				++++	25	++	++++	
				++++	30	+++	++++	
				++++	30	+++	++++	
				++++	35	++++	++++	
				++++	20	+++	++	
				++	20	+++	+++	
				—	—	++	++	
				—	—	++	++	Bad slide
V	16 d.	47 hrs.	NC	+++++	30	++++	++	
				+++++	30	++	++++	
				+++++	35	+++	++++	
				++++	30	++++	++++	
				++++	30	++++	++++	
				++	15	+	+	
				++	25	+	+	
				++	20	+	+	
				++	10	+	+	
				++	25	++	++	
				++	20	+	++	
				++	15	+	++	
			20 mg. % B ₁	++	15	+	+	
				+	10	+	+	
				+	10	++	++	
				+++++	40	++	++++	
				+++++	30	+	++	
				++++	20	++	++++	
				++++	40	++	++++	
				++++	20	++	++++	
				++++	20	++	++++	
				++	15	++	++	
				++	25	++	++	
				++	20	+	++++	
				++	20	++	++	
				++	10	+	+	
				++	20	+++	++	
				++	25	+++	+++	
				—	—	+	++	Ganglion lost in staining
VI	11 d.	42 hrs.	NC	+++++	40	++++	++++	
				+++++	30	+++	++++	
				+++++	40	++	++++	
				+++++	40	++	++	
				+++++	40	+++	++++	
				+++	30	+++	++	
				++	40	++++	++++	
			20 mg. % B ₁	+++++	45	+++	++	
				+++++	40	++	++++	
				+++++	40	++++	++++	
				++++	30	++++	++++	
				++++	30	++++	++++	
				++++	40	++++	++++	
				+++	25	++	++	

TABLE 1 (Continued)

EXP. NO.	AGE CHICK	INCUBATION TIME	CLASS	NF	L	FB	MC	REMARKS
VII	9 d.	36 hrs.	NC	++++	40	++++	++++	
				++++	25	++++	++++	
				++++	30	++	++++	
				++++	30	+++	++++	
				+++	25	+++	++++	
				+++	15	+	+++	
			24 mg. % B ₁	++++	30	++++	++++	
				++++	30	++++	++++	
				++++	45	+++	+++	
				+++	30	++++	++++	
				+++	20	+++	+++	
			NC	++++	35	++++	+++	
				+++	25	+++	+++	
				+++	30	+++	++++	
				+++	30	+++	++++	
				+++	15	++	++	
			24 mg. % B ₁	++++	20	+++	++	
				++++	20	++	+++	
				++++	25	+++	+++	
				++++	30	+++	++++	
VIII	14 d.	36 hrs.		+++	30	++++	++++	
				+++	35	+++	++++	
				+++				
				+++				
				+++				

Explanation of abbreviations used: NC, normal controls; NF, nerve fiber density; L, nerve fiber length in micrometer units; FB, fibroblast density; MC, macrophage density.

There is extreme variation not only among groups of experiments but within any one series of ganglia. It is believed that this variation is due to differences between the individual ganglia of chicks and to differences between the various chick embryos which were used as donors. In view of the small numbers of chicks used, it is impossible to make generalizations as to the effect of the age of the donor. The younger ganglia tended to spread out more over the surface of the plasma clots, and in preliminary experiments it was found that growth very rarely occurred from ganglia of chick embryos 17 days old or older. As these were the only consistent effects attributable to age which were noted, age can be ruled out in analyzing the data.

Incubation time does not affect the results within the limits of this experiment. Also, a preliminary statistical analysis showed no significant differences between the effects of the two concentration ranges of thiamin used. Consequently, the cultures have been treated as a population consisting of two classes, normal controls and thiamin-treated cultures, in the analysis which follows (see table 2).

The method of preparing the ganglia for explanation also has a profound effect on the type of growth which results. While a certain amount of variation is unavoidable in any technique where each ganglion is

TABLE 2
Analysis of grouped data.

A. Density of nerve fibers

CLASS	NORMAL CONTROLS	B ₁ -TREATED	SUM	CHI SQUARE
1	3	1	4	1.00
2	18	13	31	0.52
3	26	26	52	0.00
4	15	20	35	1.03
—	—	—	—	—
Sum	62	60	122	2.55

P is about 0.50

B. Length of nerve fibers

CLASS	NORMAL CONTROLS	B ₁ -TREATED	SUM	CHI SQUARE
10-19	13	11	24	0.16
20-29	22	22	44	0.09
30-39	18	15	33	0.12
40-49	9	10	19	0.21
—	—	—	—	—
Sum	62	58	120	0.58

NB: The 0-9 class was omitted because of insufficient numbers
P is about 0.80

C. Means and standard deviations for length of nerve fibers were calculated as follows:

	NORMAL CONTROLS	B ₁ -TREATED
Mean	25.40 micr. units	25.51 micr. units
S. D.	9.00 micr. units	9.80 micr. units

Converting micrometer units to millimeters:

	NORMAL CONTROLS	B ₁ -TREATED
Mean	0.41 mm.	0.41 mm.
S. D.	0.15 mm.	0.16 mm.

P is 1.00

D. Density of fibroblasts

CLASS	NORMAL CONTROLS	B ₁ -TREATED	SUM	CHI SQUARE
1	17	9	26	2.46
2	19	25	44	0.82
3	28	23	51	0.31
4	9	14	23	1.57
—	—	—	—	—
Sum	73	71	144	5.16

NB: 0 class omitted because of insufficient numbers
P is about 0.15

E. Density of macrophages

CLASS	NORMAL CONTROLS	B ₁ -TREATED	SUM	CHI SQUARE
1	13	6	19	1.90
2	18	18	36	0.11
3	28	25	53	0.08
4	14	20	34	1.06
—	—	—	—	—
Sum	73	69	142	3.15

NB: 0 class omitted because of insufficient numbers
P is about 0.40

prepared individually, all the ganglia used in any experiment were removed before any were cultured, and the ganglia which were to serve as normal controls and as experimentals were selected at random from the mixed lot.

While in most cases a fairly close correlation exists between the various cell components growing out of the spinal ganglia, this correlation is not invariable. Thus a few cultures show a dense growth of fibroblasts but few nerve fibers and vice versa. Length and density of nerve fibers are also not invariably correlated. Probably not too much significance should be attached to the numbers of macrophages present, because they originate partly from embryonic blood vessels attached to the ganglia (particularly in those from the older embryos) and partly from transformation of sheath cells (Weiss, unpublished observations). Hence, the numbers originally present vary considerably with small changes in the method of preparation.

DISCUSSION

It would seem from these data that any differences between nerve fiber length and density in normal and experimental cultures and between macrophage density in the two types of cultures might be due as well to chance variation as to the effects of the vitamin. While the figures on fibroblasts may possibly indicate a trend towards stimulation by the vitamin, the difference is not statistically significant.

Several assumptions are implicit in this work and should be discussed briefly. First, it is assumed that thiamin persists long enough in the medium to exert whatever biological effects it may have. It is well established that the thiamin molecule will break down into two biologically inactive fractions (as far as animal metabolism is concerned) if it is kept in an alkaline solution. However, as free thiamin has been demonstrated in various tissues of the body which are normally slightly alkaline, it is believed that this assumption is justified. Also it is assumed that thiamin can be converted to cocarboxylase by the cells of the spinal ganglia. This has not been shown in tissue culture, but Peters and his associates have demonstrated it convincingly in the case of brain cell suspensions *in vitro*. Thirdly, it is assumed that the stimulating effects would be perceptible within the time of short-range tissue culture. While many of the effects of vitamin B₁ are apparent within a few hours, this point is being clarified by more extended periods of cultivation.

The significance of these findings for the study of nerve regeneration is obvious because any substance which would increase the growth rate

of fibers would be of great value in neurosurgery. It has not been shown that vitamin B₁ is such a substance. However, the conclusions of Ward and Kennard and of Tangari as to the value of B₁ in trauma would by no means be invalidated by a demonstration that thiamin chloride does not speed up the rate of actual morphological regeneration in the intact organism. On the contrary, it would make Ward and Kennard's hypothesis as to the indirect role of thiamin by way of the neuro-humoral system seem more suggestive.

As may be seen from the literature reviewed earlier in this paper, it is not necessary to assume a growth stimulating role for thiamin to explain most of its reported effects. Thus avitaminotic symptoms are usually relieved by thiamin within a few hours, much faster than could be accounted for by any structural changes within the nervous system. The clinicians who have obtained positive results with the vitamin usually report them as occurring within a very short time after administration.

Most of the studies on the effects of vitamin B₁ on growth have compared animals receiving thiamin with those on a deficient diet, so that, while they demonstrate that thiamin is essential for growth, they are not evidence that excess thiamin will stimulate growth beyond normal physiological limits. The weight increases in rats reported by Whipple and Church are caused by fat accumulation rather than true growth.

In conclusion, while it is not possible to draw too close analogies between various classes of organisms, it is interesting to note that the spinal ganglia studied here seem to resemble many species of bacteria and the roots of many plants in that thiamin, in concentrations above the normal physiological range and below the limits of toxicity, has no effect on growth in tissue culture.

SUMMARY

1. Spinal ganglia of 9- to 16-day embryonic chicks have been cultured for 2 days in media to which various concentrations of thiamin chloride were added and compared with control cultures grown without added vitamin.

2. No significant differences attributable to the influence of thiamin were found between control and experimental cultures in length and density of nerve fiber outgrowth, fibroblast density, and macrophage density when the concentrations of thiamin used were below the threshold of toxicity.

3. It is concluded that vitamin B₁, between the normal physiological range and the limits of toxicity has no effect on axon growth in tissue culture. The significance of this finding for nerve regeneration is discussed.

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NEURULATION IN MECHANICALLY AND CHEMICALLY INHIBITED AMBLYSTOMA

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INTRODUCTION

Although the dependence of the medullary plate upon the chorda-mesoderm has attracted considerable attention from embryologists, the mechanism by which the plate becomes a neural tube has not been demonstrated.

In amphibians, it has been claimed that pressure exerted by ectoderm and mesoderm (Giersberg, 1924) or by the liquid confined between those two germ layers (Ruffini, 1925) is an active factor in neurulation. However, Lehmann (1926) and Boerema (1929), using different experimental approaches, have demonstrated that neurulation in these forms is an autonomous process within the medullary plate. In echinoderms (Moore and Burt, 1939; Moore, 1941) gastrular invagination, which in many respects resembles neurulation, has likewise been shown to be independent of ectodermal pressure.

Mitosis accompanied by a differential increase in cell volume has also been thought to be a factor in neurulation. Although little or no mitotic activity during this process was found by Glaser (1914) in *Cryptobranchus alleghehniensis* or by Ruffini (1925) in Triton, the latter worker believes mitosis to be a contributing factor to neurulation in Rana. Derrick (1937) reports that the high mitotic rate in the sides of the chick medullary plate as compared with the floor may aid neurulation in that form. In this animal it has also been found that after the neural tube has closed, incidence of mitosis is higher in the evaginating optic vesicles than it is in other regions of the brain (Frank, 1925). Hutchinson (1940), on the other hand, finds that the elongation of the neural tube which occurs soon after its closure in Amblystoma is not due to cell proliferation.

The hypothesis of Glaser (1914) that neurulation in *Cryptobranchus* may be caused by differential water absorption in the medullary plate cells has not been supported by the data of Brown, Hamburger, and Schmitt (1941) on Amblystoma. They find no appreciable increase in the water content of the plate during the critical period as determined by density measurements. Hobson (1941), however, was able to produce unfolding of partially closed chick neural tubes by dehydrating them in hypertonic media.

Ruffini (1925) reports that neurulation is aided by autonomous, amoeboid motion of the medullary plate cells. Boerema (1929) concludes that autonomous changes in cell shape are the responsible mechanism. It is well established (Goerttler, 1925; Vogt, 1929; Manchot, 1929, and many others) that extensive

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cell movements take place within the neural ectoderm which result in the elongation of the structure, but it is not known to what extent these movements are correlated with the formation of the neural tube.

It was the purpose of the work reported here to compare the cellular changes taking place in normal embryos during neurulation with those in embryos in which neurulation had been inhibited by various means in an attempt to find some clue to the factors responsible.

MATERIALS AND METHODS

Several clutches of eggs of *Amblystoma maculatum* (Shaw) and of *Amblystoma tigrinum* (Green) were used, some of which were obtained near Chicago and some of which were shipped from Pennsylvania. The eggs were reared at room temperature unless otherwise noted, and care was taken that environmental conditions should be the same for experimentals and controls in a given series. Stage numbers of all specimens refer to Harrison's tables (1918, unpublished).

Most of the embryos were fixed in modified Formol-Zenker, double embedded in celloidin and paraffin, and sectioned at 6 micra. Some specimens were stained with Ehrlich's hematoxylin and mucicarmin for the study of cell shape, nuclei, and pigment granules; others were stained with neutral gentian violet to differentiate yolk and secretion granules. A few embryos were fixed in picric alcohol and stained with Best's carmine for the determination of glycogen.

MECHANICAL INHIBITION OF NEURULATION

Firstly, mechanical inhibition of neurulation was accomplished as follows: The medullary plates plus underlying mesoderm were excised from each of two *Amblystoma maculatum* embryos in Harrison's Stage 12 and explanted into Holtfreter's solution. One plate was then placed on top of the other and the two pieces of tissue weighed down with splinters of cover glass in such a manner that the plates could not fold up to form a tube. In some cases the plates were oriented so that the ectoderm of one was in contact with the mesoderm of the other; in other cases ectoderm was in contact with ectoderm. Six double explants of this type were studied. A number of intact *Amblystoma* eggs from the same clutch from which the membranes had been removed were reared in Holtfreter's solution, and 10 explanted medullary plates were allowed to develop freely in the same medium as controls.

The unoperated eggs developed normally except that, in some cases, the hypertonic medium caused a slight retardation of the head region. By the time the normal controls had reached Stage 28, the free explants showed distinct signs of neurulation. When the normal controls were in Stage 31 (Plate I, Fig. 1), the free explants had prominent neural folds which in some cases had nearly closed to form a tube (Plate I, Fig. 2). At the same time in the weighted explants, the medullary cells had elongated and become columnar as in early stages of normal neurulation, but the flask shape characteristic of later stages was never assumed and a tube was not formed (Plate I, Fig. 3).

There was no apparent difference in cell shape or intracellular organization between weighted explants whose ectoderm was in contact with ectoderm and those whose ectoderm was in contact with mesoderm. Thus it would seem that

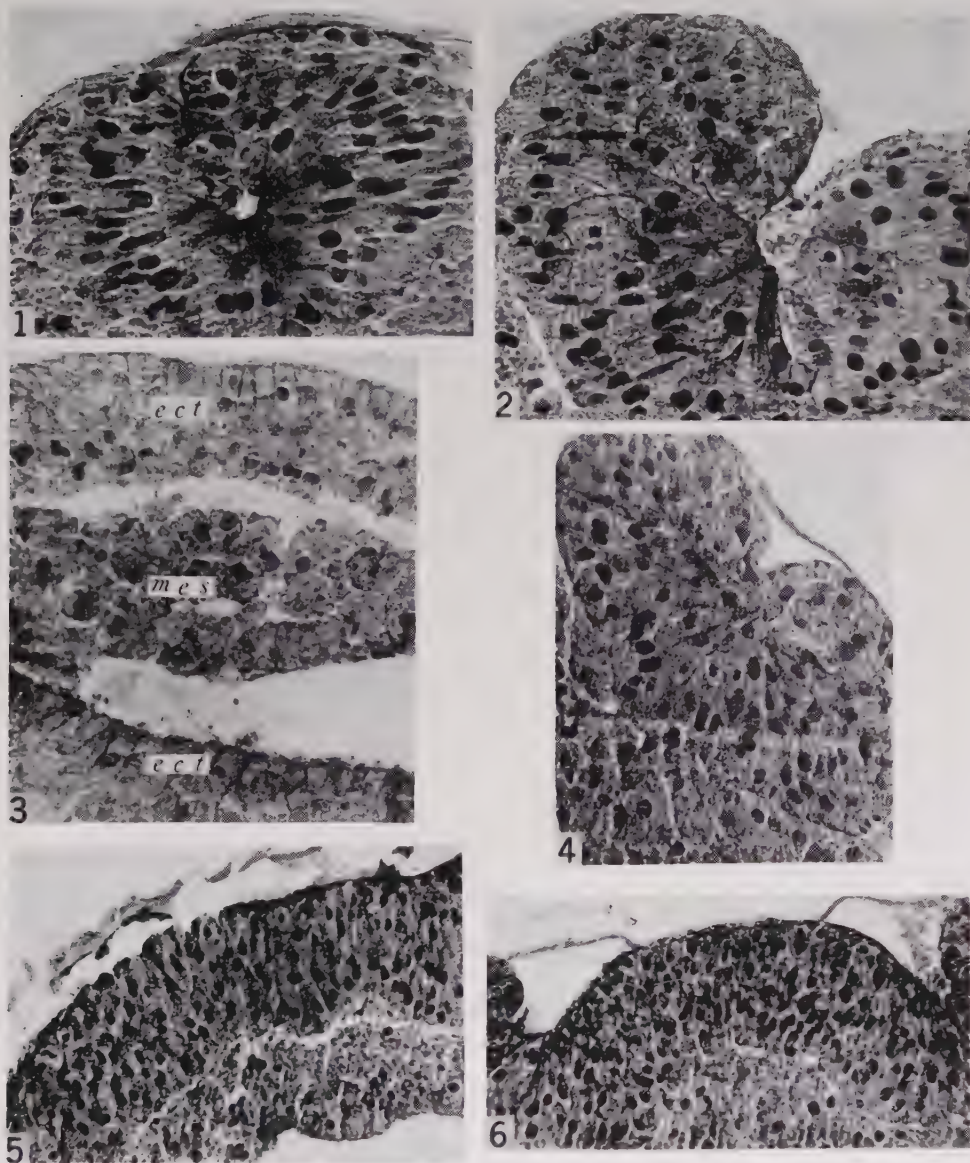


PLATE I

FIGURE 1. Neural tube of normal *A. maculatum* embryo. 250 $\times$.

FIGURE 2. Medullary plate from embryo of same chronological age as Figure 1 explanted into Holtfrete's solution. 250 $\times$.

FIGURE 3. Double explant, same age as Figure 1. ect. = neural ectoderm. mes. = mesoderm. 250 $\times$.

FIGURE 4. Normal *A. tigrinum*, Stage 18. 250 $\times$.

FIGURE 5. Ringer-treated embryo, same chronological age as Figure 4. 160 $\times$.

FIGURE 6. LiCl-treated embryo, same chronological age as Figure 4. 160 $\times$.

by Stage 12 the dorso-ventral polarity of the neural plate has already been established. In both the weighted and free explants the cells were rounder and shorter than those in the controls, the nuclei were round as compared to the oval ones in the normal animals, and there was a heavier deposit of pigment granules around the distal edges of the medullary cells. No other significant differences were noted.

From these data it was concluded that, while pressure at right angles to the plane of the medullary plate can inhibit closure of the neural folds, it does not suppress the initial cell elongation which accompanies that closure.

CHEMICAL INHIBITION OF NEURULATION

Next the developing eggs were subjected to the action of lithium chloride and of hypertonic salt solutions which, in the proper concentrations, will produce delayed closing of the neural tube or permanent *spina bifida*. Three series of experiments were carried out.

TABLE I

Comparison of development of normal, LiCl- and Ringer-treated Amblystoma. Figures refer to Harrison's Stages

	Normal controls	M/10 LiCl	Mammalian Ringer's	Remarks
<i>Series 1</i>				
<i>A. tigrinum</i>	19	15	16	
20° C.	29-30	16	18-19	
	34-35	18-19	20-21	neural tube still open in head region
<i>Series 2</i>				
<i>A. maculatum</i>	16-18	13	12	
12° C.	19-20	15	12	
	22-23	18-19	—	died about 140 hours after immersion in salt solution

The first series consisted of three groups of 33 *Amblystoma tigrinum* eggs which at the inception of the experiment were in Stage 13. The first group were reared in well water to serve as normal controls. The second group were reared in M/10 LiCl solution, the third in mammalian Ringer's. A second series consisted of three groups of 17 *A. maculatum* eggs which at the beginning of the experiment were in Stage 12 b. As with the *tigrinum* eggs, one group was reared in well water, one in M/10 LiCl, and one in mammalian Ringer's. However, the *maculatum* eggs, instead of being kept at room temperature, were placed on a water table with a practically constant temperature of 12° C.

The nervous systems of the treated animals in both series diverged considerably from the mean of normal development. In general, the head region was more retarded than the spinal cord. The approximate degree of maturity attained by the experimentals in comparison with the controls is shown in Table I. In staging the treated animals, external appearance was the criterion used.

It should be noted that the difference between the normal and lithium-treated embryos is greater at 20° C. than at 12° C. (this confirms the work of Hall (1942)

on *Rana pipiens*) but that low temperatures apparently augment the effect of Ringer's solution.

The third series consisted of three groups of 17 *A. tigrinum* eggs which were in Stage 11 b – 12 a at the beginning of the experiment. One group served as normal controls, one group was immersed in M/20 LiCl for 24 hours, after which development was allowed to continue in well water, and the third group was similarly treated with M/20 NaCl. NaCl treatment had no perceptible effect on the rate or type of development, while the equimolar LiCl solution retarded the embryos considerably. This series of eggs was fixed in picric alcohol for a rough determination of glycogen content.

Effects of chemical inhibition on mitotic rate

The effects of chemical inhibition were best seen in the first series of eggs as the Ringer-treated eggs did not develop at all in the second series. One-third of

TABLE II

Mitotic rate in the medullary plate of A. tigrinum. Stage numbers not in parenthesis refer to normal controls; those in parenthesis refer to inhibited animals of the same chronological age as the normal controls

	Stage	Cells counted	Mitoses seen	Mitotic index
Normal controls	18	1461	39	2.67%
	30	776	29	3.73%
	35	1037	42	4.05%
Lithium chloride-treated	18(15)	1970	19	0.96%
	30(16)	1109	12	1.08%
	35(18)	693	3	0.43%
Ringer's treated	18(16)	2276	28	1.23%
	30(18)	760	18	2.37%
	35(20)	1074	30	2.79%

the embryos were fixed and sectioned when the normal controls were in Stage 18 (at which time the normal germs had open medullary plates with well raised neural folds), one-third when the controls were in Stage 30, and the remainder when the controls were in Stage 35, by which time the lithium embryos were in approximately the same stage of development as the controls at Stage 18 as far as external appearance was concerned, and the Ringer-treated germs were slightly more mature. The effect of this inhibition on the mitotic rate in the medullary plate is summarized in Table II.

From this it is apparent first, that there is mitosis in the neural plate of *A. tigrinum* during neurulation; secondly, that the mitotic rate rises in the normal animal after the neural tube is closed; thirdly, that Ringer's solution depresses the mitotic rate in comparison with normal embryos of the same chronological age, but that the mitotic rate in Ringer-treated animals is comparable to that in normals of the same stage of development, and fourthly, that LiCl causes both a relative and an absolute decrease in the mitotic rate of the neural tube.

Effects of chemical inhibition on cell shape

The effects of inhibition on the cellular morphology of the neural tube were extreme. When the normal controls were in Stage 18 (Plate I, Fig. 4), the Ringer-treated animals showed a slight evagination of the floor of the medullary plate (Plate I, Fig. 5), and lithium-treated embryos a very marked evagination (Plate I, Fig. 6).

By the time the normal controls were in Stage 30 (Plate II, Fig. 7), ectoderm had begun to grow over the edges of the plate in the Ringer-treated germs and a slight invagination of the plate was present (Plate II, Fig. 8). When the controls were in Stage 35 (Plate II, Fig. 10) and the Ringer-treated embryos in what corresponded to Stage 20 in the normal animals, the invagination was fairly deep in the treated germs and the edges of the plate were raised, although they were not bent over as normal neural folds are at that time (Plate II, Fig. 11).

In the LiCl-treated germs, on the other hand, when the controls were in Stage 30, a flat plate was present (Plate II, Fig. 9). When the controls had reached Stage 35 and the lithium-treated animals were in Stage 18 as far as external appearance was concerned, the neural plate was still flat, but a few flask-shaped cells had appeared at the edges as in the first stages of normal neurulation. Many of the medullary plate cells in these embryos, particularly in the head region, became round and sloughed off into the space above the plate (Plate II, Fig. 12). Child (1941) reports a similar dissociation of the endodermal plate in the starfish, *Pateria*, when exposed to the action of lithium chloride.

The changes in shape occurring in both the normal and treated embryos naturally correspond to the changes in the shape of the plate as a whole. These changes may be summarized by saying that both Ringer and LiCl treatment produce, first, a more or less evaginated medullary plate and then a flat or slightly invaginated plate which may, according to the concentration of the chemicals used, proceed to form a tube in places or to be overgrown by ectoderm, and that no traces of a neural keel as described by Baker (1927) were seen in the treated embryos in the series studied.

Effects of chemical inhibition on nuclear size

Much importance has been attached to changes in cell and nuclear size during neurulation since Glaser (1914) found that, in *Cryptobranchus*, the volume of the neural plate increased during the course of neurulation and believed that this indicated an increasing water content of the neural plate. He also inferred that increased hydration occurs during gastrulation in echinoderms because of reported increases in nuclear size during that process. As Brown, Hamburger, and Schmitt (1941) found no indications of increased hydration in density measurements on *Amblystoma*, an effort was made to throw more light on the problem by measuring the nuclear axes of 100 medullary plate cells in both normals and experimentals in each of three stages. As these nuclei are not perfect spheres and as their orientation varies somewhat within the plate, these measurements cannot be used to calculate nuclear volume. However, any large changes in nuclear volume should be revealed by this method. Indices of nuclear area and shape were also calculated. These data are summarized in Table III.

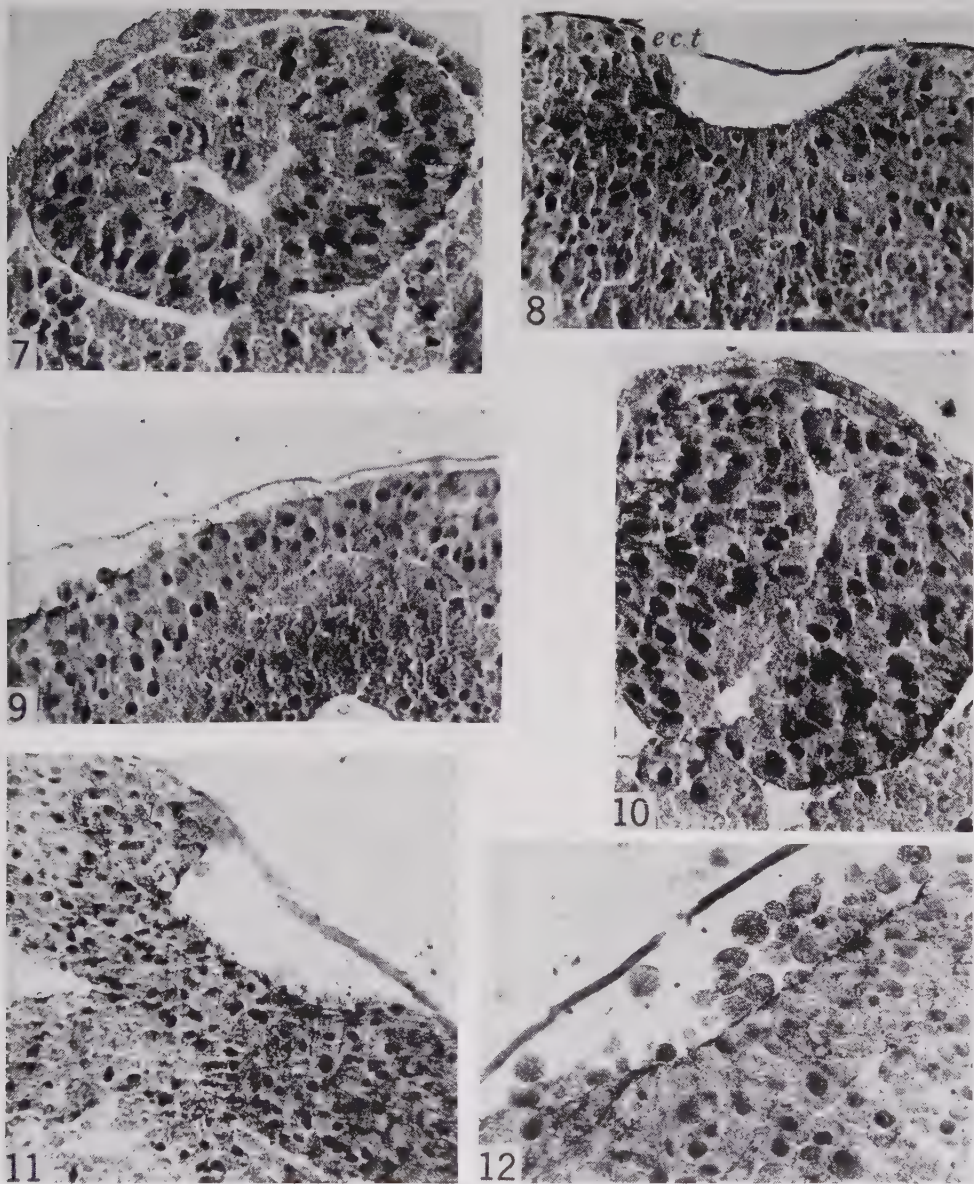


PLATE II

FIGURE 7. Normal *A. tigrinum* embryo, Stage 30. 250 $\times$.

FIGURE 8. Ringer-treated embryo, same chronological age as Figure 7. ect. = ectoderm growing over medullary plate. 160 $\times$.

FIGURE 9. LiCl-treated embryo, same chronological age as Figure 7. 160 $\times$.

FIGURE 10. Normal *A. tigrinum* embryo, Stage 35. 250 $\times$.

FIGURE 11. Ringer-treated embryo, same chronological age as Figure 10. 160 $\times$.

FIGURE 12. LiCl-treated embryo, same chronological age as Figure 10. Note round cells sloughed off from plate. 250 $\times$.

No statistically significant differences in nuclear axes, area, or shape were revealed by this analysis between normal and treated nuclei because of the large standard deviations involved. However, it should be noted that the index of shape (A/B) increased consistently in the normal germs whereas it remained practically constant or decreased slightly in the chemically treated germs. This lack of nuclear elongation seems to be correlated with the failure of cell elongation which was also observed in these cases. While no conclusions can be drawn from these findings as to cellular hydration, there is no change of nuclear size during folding of the neural plate.

Effects of chemical inhibition on cellular inclusions

A. *Yolk granules.* In normal *A. tigrinum* and *A. maculatum* embryos, yolk begins to be utilized in the neural tube, beginning in the head region, about

TABLE III

Greatest nuclear length (A) and diameter (B) of 100 medullary plate cells. Stage numbers not in parenthesis refer to normal controls; those in parenthesis refer to treated animals. All measurements are in ocular micrometer units

	Stage	Nuclear measurements				Indices			
		Length (A)		Diameter (B)		Area (AB)		Shape (A/B)	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Normal controls	18	15.00	2.22	8.84	1.58	129.4	34.8	1.72	0.35
	30	14.60	2.76	8.42	1.56	119.2	30.6	1.80	0.51
	35	16.80	2.83	8.86	1.52	146.6	36.8	1.93	0.48
Ringer-treated	18(16)	13.55	2.41	8.66	1.56	115.9	33.4	1.59	0.38
	30(18)	14.33	2.24	9.03	1.71	126.7	38.8	1.58	0.36
	35(20)	13.27	2.18	9.42	1.56	121.7	32.2	1.42	0.32
LiCl-treated	18(15)	14.79	2.21	9.51	1.46	139.7	34.4	1.45	0.27
	30(16)	14.63	2.22	10.79	1.56	157.3	38.0	1.26	0.25
	35(18)	13.96	2.03	10.02	1.17	138.7	32.0	1.38	0.20

Stage 18. By Stage 35, yolk has practically disappeared from the brain, and only a sparse scattering of granules remains around the lumen of the spinal cord. Bragg (1939), who has studied the utilization of yolk in a number of other amphibian genera, reports that in his animals it did not begin until after the closure of the neural tube.

Lithium chloride and Ringer's solution both seem to retard the disappearance of yolk as well as the closure of the neural tube, as the medullary plates of the treated animals were packed with yolk granules throughout the period of observation whereas, in the normal controls, the amount significantly diminished. It is doubtful if this is causally related to the process of neurulation, however, because (1) yolk disappears very late in this process and (2), as Morgan (1906) has shown, eggs of *Bufo variabilis* centrifuged so that all granules are thrown out of portions of the head in the resultant embryos will develop closed neural tubes.

B. *Glycogen*. In the *A. tigrinum* series, no perceptible change in the glycogen content of the nervous system was noted between Stage 18, at which time the neural tube is open over its full length, and Stage 30, when the entire tube is closed and morphogenesis of the brain is well under way. All the neural cells contained much glycogen, no significant differences being noted among the various regions of the nervous system.

Treatment with M/20 NaCl did not affect glycogen distribution (as was to be expected since no morphological changes were observed) nor did treatment with M/20 LiCl. Thus, although no histological method is exact enough to reveal very small changes in glycogen content, it would appear that in *A. tigrinum* neurulation is not accompanied by significant utilization of this material.

C. *Pigment granules*. Early in the normal process of neurulation in Amblystoma, as reported by Ruffini (1925) and Lehmann (1926) for other urodeles, there is a marked accumulation of pigment, especially in creases formed by the medullary folds. From the pigment layer at the distal ends of the cells, rows of pigment granules extend along the cell boundaries (see Plate I, Fig. 1). When neurulation is completed, there is a layer of pigment granules along both surfaces of the neural tube and many granules along the cell boundaries and within the cell bodies.

The chief difference noted in the treated animals was that the concentration of pigment near the outer surface of the plate cells occurred irregularly and only in those cells where shrinkage of the inner surface took place (see Plate I, Figs. 5 and 6; Plate II, Figs. 8 and 12). In the lithium-treated specimens, as soon as degeneration of the plate commenced, many granules escaped into the free space above the plate, and all of the sloughed-off cells were packed with pigment. These granules, like yolk, however, appear to be a passive factor in neurulation and are of value only as an indicator of the results of active processes which change cell shape.

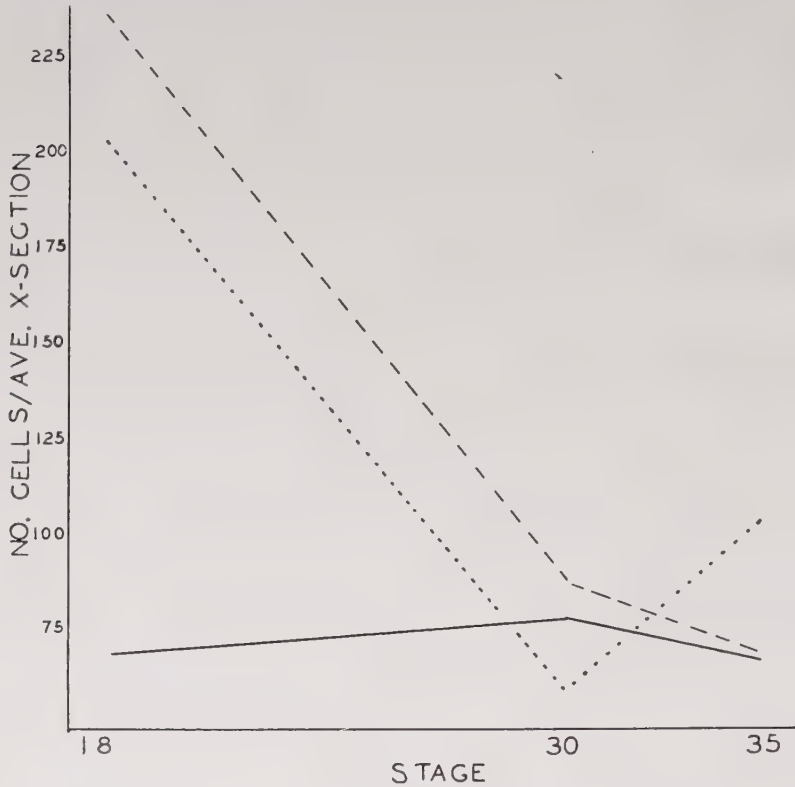
D. *Secretion granules*. As shown by Studnička (1900) and Weiss (1934), secretion occurs in the embryonic ependyma. On the chance that secretion might be involved in the process of neurulation, normal and chemically inhibited *A. tigrinum*, *A. maculatum*, and *Rana pipiens* germs and normal chick embryos which had been stained with neutral gentian violet were examined for secretion granules. None were found, either in the normal embryos or in the treated amphibians. This does not necessarily indicate that unformed secretion does not occur; in fact, the presence of liquid within the lumen of the neural tube is evidence that secretion of some sort does take place very early in normal development. Because of the difficulty of demonstrating secretion antecedents, however, the problem requires study by more refined techniques.

Effects of chemical treatment on cellular movements

The most accurate method of following cell movements in embryonic development is by vital staining, a procedure not used in this investigation. However, some indication of those movements was obtained by counting the number of cells in the neural plate in every fifth section of the trunk region of embryos (exclusive of brain and tail) and averaging the results. It is very hard to obtain strictly comparable results by this method because, as Manchot (1929) has shown, in the normal development of urodele embryos, the anterior two-thirds of the neural

plate becomes brain while the posterior one-third elongates to form the spinal cord. A rough comparison between the normal and chemically treated neural plates at various stages of development is presented in Graph 1.

As this graph was constructed from data on only nine animals, not too much significance can be attached to it. However, it would seem that in normal animals between Stages 18 and 35 mitosis and stretching of the neural plate keep pace with one another so that the average number of cells per cross section remains



GRAPH 1. Average numbers of cells present in every fifth section of the medullary plate of normal and chemically treated *A. tigrinum* embryos of the same chronological age. Abscissae are stage numbers of normal controls. Solid line used for normal controls, dashes for lithium embryos, dots for Ringer germs.

approximately constant and that, although LiCl and Ringer's solution do inhibit the stretching process just as they inhibit neurulation and mitosis, elongation of the neural plate continues under their influence.²

² In Glaser's work on *Cryptobranchus* (1914), he used the average number of nuclei present per cross-section to test whether or not cell division was occurring. Because the number of nuclei remained approximately constant, as it does in normal *Amblystoma* during slightly later stages, he concluded that there was little or no mitosis during neurulation. It would be interesting to re-examine his material to see if elongation of the medullary plate played any role in that constancy.

DISCUSSION

In discussing neurulation, it must be borne in mind that the folding of the neural plate is a very complex process involving not only changes in cell size and proportions, but physical and biochemical changes which have, as yet, been little studied. From the foregoing analysis, it can be concluded that certain factors are not involved in the more obvious phases of folding. Thus not only does neurulation occur, as has been previously reported by many workers, in the absence of normal mechanical pressures, but the characteristic preliminary cell elongation takes place when pressure is exerted at right angles to the usual direction of cell movement.

Neurulation seems to be independent of nuclear area, and if, as has been suggested, the latter be accepted as an index of cell hydration, also independent of hydration. For, although treatment with Ringer's solution and LiCl had no significant effect on nuclear area, it did inhibit folding of the medullary plate. On the other hand, nuclear elongation, which accompanies cell elongation in the normal plate, does not occur when folding is inhibited.

Judging by the data on average number of cells per section, the elongation of the medullary plate which normally takes place during and after neurulation is not necessarily correlated with the closure of the neural tube, because, although both LiCl and Ringer's solution do retard the stretching process somewhat, it continues even when neural folds do not form.

Mitosis seems to be either directly instrumental in neurulation or at least under control of the mechanism of neurulation. Thus in the LiCl-treated embryos, in which the mitotic rate fell to a very low value during the experiment, no folds appeared, while the elevation of the sides of the neural plate in both the normal and the Ringer-treated specimens was accompanied by active cell division.

Cell elongation and wedging are unavoidably correlated with embryonic folding, and, as Boerema (1929) and others have pointed out, such changes are theoretically quite sufficient to cause neurulation. As demonstrated by Brown, Hamburger, and Schmitt (1941) differential water absorption cannot account for such changes in *Amblystoma*. These workers and Schmitt (1941) independently have suggested that molecular interactions and desolvations in the cell surface may exert the forces necessary to cause cell elongation. Weiss (unpublished) has suggested further that the concentration of pigment granules which occurs in the normal folding plate indicates a contraction of the cell cortex at the free surface. Although Hobson (1941) has not succeeded in demonstrating any systematic changes in the ultrastructure of the chick neural plate during folding by polariscopic analysis, a more intensive investigation of such changes during neurulation appears to be the most promising method of approach to the problem.

An interesting point which emerges in a comparison of the LiCl and Ringer-treated germs is that the effects of the two agents on neurulation seem to be produced by different means. Thus lithium is less effective at low temperatures, while hypertonic salt solutions are more effective. Further, lithium salts inhibit neurulation at much lower concentrations than do those present in Ringer's solution. Hall (1942) has evidence that lithium is a toxic agent acting on the chordamesoderm rather than on the responding ectoderm. The fact that Ringer's solution is a more effective inhibitor at low than at high temperatures would suggest that it acts on the physical consistency of the embryo rather than on chemical

processes, perhaps by stiffening the neural plate so that folding is impeded—a suggestion which Giersberg (1924) has previously offered to explain the action of sucrose and sodium acetate on neurulation.

Finally, it should be noted that the data presented here are by no means conclusive in themselves; they are offered merely in an effort to shed light on a few phases of a very complicated problem.

SUMMARY

1. Mechanical pressure exerted at right angles to the plane of explanted medullary plates has been found to suppress neurulation in *Amblystoma maculatum*, but not the preliminary cellular elongation which is normally involved in that process. This elongation takes place irrespective of whether the medullary plate is in contact with ectoderm or with mesoderm on the normally free surface.

2. In *Amblystoma tigrinum* and *A. maculatum* neurulation is accompanied by mitosis, the mitotic rate rising after the neural tube has closed. Treatment with mammalian Ringer's solution at room temperature decreases the mitotic rate to about the same degree as it inhibits normal development; treatment with M/10 LiCl decreases the mitotic rate both relatively and absolutely.

3. No statistically significant difference was found in average nuclear area between normal and treated medullary plates. In normal germs, the nuclei elongate during neurulation, whereas in the treated germs they did not.

4. Glycogen and yolk begin to disappear from the normal neural tube about Stage 18. Neurulation-inhibiting chemicals retard the utilization of these substances.

5. Pigment granules appear to be passive factors in neurulation indicative of contraction at free cell surfaces.

6. No evidence of formed secretion from the neural plate was found.

7. Although inhibiting chemicals decrease the rate of elongation of the medullary plate, stretching continues even when neural folds fail to form.

8. The inhibiting action of LiCl is less effective at low temperatures, that of Ringer's is augmented.

9. It is concluded that neurulation in *Amblystoma* is autonomous to the medullary plate and may be aided by mitotic activity; changes in nuclear area (which may be indicative of cell hydration), intracellular inclusions, and longitudinal cell movements are not instrumental in the process.

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GROWTH OF SPINAL GANGLIA IN PLASMA FROM VITAMIN B₁-DEFICIENT CHICKENS

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ONE FIGURE

INTRODUCTION

As reported previously (Burt, '43), addition of thiamin chloride to tissue culture media produces no statistically significant increase in nerve outgrowth from spinal ganglia of chick embryos. As a supplement to this work, the growth of ganglia in plasma taken from chickens in the last stages of vitamin B₁-deficiency has been studied.

The effects of lack of thiamin on the nervous system of the intact animal have been reviewed in the paper referred to above; however, the only work on the growth of nervous tissue in plasma from polyneuritic fowls is that of McCarrison and Sankaran ('33), who report that in cultures of 8 to 9-day chick embryonic spinal cord, very few nerve fibers appear and those that do regress more rapidly than do fibers in control cultures made with normal plasma. They also find that chick heart fibroblasts, when cultured in deficient plasma, proliferate less rapidly and are smaller, longer, and thinner than the typically fusiform fibroblasts of normal cultures. As subcultures of spinal cord from B₁-deficient into B₁-deficient plasma undergo very rapid regression, McCarrison and Sankaran believe this medium to be actively toxic for nerve tissue.

In animals suffering from B₁ deficiency, there is known to be an accumulation of intermediary compounds involved in carbohydrate metabolism, but the evidence for possible toxicity of these compounds is conflicting. Thus the pyruvic acid level is elevated in the blood of B₁-deficient pigeons (Johnson, '36; Lu, '39a), rats, rabbits, and men (Lu, '39a). In rats (Lu, '39b), the bradycardia which accompanies B₁-deficiency is paralleled by a rise in blood pyruvate, and in human

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beri-beri patients, severe exercise, which raises the blood pyruvate level, often precipitates attacks of the disease (Lu and Platt, '39). However, injection of pyruvate has no effect on the heart rate of the rabbit and in the rat produces lethal convulsions rather than the symptoms of B₁-deficiency.

Shils, Day, and McCollum ('41) report that excretion of bisulfite binding substances ("b.b.s."), which they believe to consist mostly of pyruvic acid, is the first objective indication of thiamin deficiency in rats. Taylor, S. Weiss, and Wilkins ('37), however, found a higher b.b.s. level in humans than could be accounted for by pyruvic acid, acetone, and diacetic acid. In man also, the b.b.s. level is frequently elevated in the absence of clinical B₁-deficiency and is apparently poorly correlated with deficiency symptoms where they do exist (Wilkins, Taylor, and S. Weiss, '37). These findings are confirmed by Wortis, Bueding, and Wilson ('40), who have likewise noted great variation of the b.b.s. level in psychiatric cases where a B₁-deficiency was not involved. Haynes and S. Weiss ('40) were unable to simulate the effects of B₁-deficiency on the rat heart by the administration of pyruvic, lactic, or adenylic acid, methyl glyoxal, or glyceraldehyde. They even observed recovery of deficient rats to which thiamin and intermediary metabolites were administered simultaneously.² Consequently, in addition to checking the effects of B₁-deficient plasma on nerve growth in tissue culture, it was deemed necessary to study the effects of some intermediary metabolites as well.

MATERIALS AND METHODS

The experimental procedure used was the same as that described in the analysis of the effects of thiamin chloride on growth in tissue culture. Lumbo-sacral spinal ganglia were dissected out of chick embryos of 9 to 15 days incubation and cultured within paraffin rings on mica. The various chemicals tested were added dissolved in the modified Tyrode's solution previously described, and the pH was adjusted to 7.4 before use by the addition of sterile 1% sodium bicarbonate, using a phenol red indicator.

B₁-deficient plasma was obtained from four Leghorn chicks 6 weeks to 3 months of age which were kept on Wisconsin diet 242A (Arnold and Elvehjem, '38) until they were unable to stand and would, as experience had shown, have died within the course of the next day. The chickens used took from 2 to 3 weeks to reach this condition after

² A more complete review of the literature on toxic compounds in B₁-deficiency may be found in the paper of Haynes and Weiss and in that of Platt and Lu ('39).

having been placed on the B₁-deficient diet. Blood was obtained by heart puncture with the use of heparin in all cases. The resulting plasma was not tested chemically for the presence of thiamin and cocarboxylase; however, for convenience the plasma from B₁-deficient fowls will be referred to as "B₁-deficient plasma" or simply as "deficient plasma", although it is realized that this implies an unproved assumption.

Observations on the incubated living cultures were made each day, and the density of various types of cells present in the growth zone was recorded. However, the presence of cellular elements in the growth zone is not necessarily indicative of "growth" in the sense of increased living mass. For example, it is not known whether the axon fibers that appear in tissue culture "grow" by taking in material from the surrounding medium or whether they represent merely pseudopodial extensions of the neurons which lie within the ganglion. Furthermore, the non-nervous elements, fibroblasts, sheath cells, and macrophages, which are observed in the growth zone, may have originated by mitosis there or may be present merely because they have migrated out of the ganglion. As it is frequently difficult to distinguish between fibroblasts and sheath cells in tissue culture, these two types of cells have been lumped together under the classification of "spindle cells."

Density of spindle cells and macrophages in the growth zone was recorded by an arbitrary system in which class O signified no cells present, class I a very sparse concentration of the element being studied, class II moderate density, class III heavy density, and class IV a very heavy density. Nerve fiber concentration was estimated after fixation and staining (Bodian's protargol method), as the living fibers are sometimes masked by other cells. The same system of grading was used for nerve fibers as for spindle cells and macrophages and has been previously illustrated (Burt, '43).

EXPERIMENTAL

Effects of B₁-deficient plasma on outgrowth from spinal ganglia

The first experiment performed to test the effects of vitamin B₁-deficient plasma on nerve growth in vitro consisted of five sets of cultures totalling 107 in all. Forty-two of these cultures were grown in normal chicken plasma and served as controls. Thirty-four were grown in deficient plasma, and thirty-one in deficient plasma to which 4 mg. per cent thiamin chloride had been added. Incubation periods ranged

from 42 hours, by which time maximum nerve growth had been attained in most cultures, to 67 hours, when regression was just beginning in the normal controls. The deficient plasma from one 6-week-old chick seemed to be more detrimental to the growth of spinal ganglia than was that from the three older chickens, but not much significance can be attached to this finding because of the small number of animals used.

A statistical analysis of the data grouped with regard to density of the various types of cells involved is presented in table 1. For several

TABLE 1
Density of cells observed in cultures of spinal ganglia after 2 to 3 days of incubation.

	CLASS	NORMAL CONTROLS	DEFICIENT PLASMA	DEFICIENT PLASMA + B ₁	CHI SQUARE	
					Controls: deficient	Deficient: deficient + B ₁
Nerve fibers	0	0	6	6	6.00	0.00
	I	2	20	16	18.35	0.11
	II	20	7	6	3.75	0.00
	III	16	1	3	8.75	1.00
	IV	4	0	0	4.00	...
	Total	42	34	31	40.85	1.11
Spindle cells	0	0	9	2	11.25	3.30
	I	5	14	10	5.28	1.68
	II	23	3	9	12.53	3.00
	III	7	3	5	1.92	0.50
	IV ¹	1	0	0		
	Total	36	29	26	30.98	8.48
Macrophages	0 ¹	1	1	0		
	I	9	10	12	0.86	0.18
	II	11	4	3	2.42	0.00
	III	13	9	6	0.18	0.27
	IV	2	5	5	2.33	0.00
	Total	36	29	26	5.79	0.45

¹ Class omitted from statistical calculations because of insufficient numbers.

reasons, all cell types were not studied in all cultures, which explains the discrepancy in total numbers of cases. As may be seen from this table, density of nerve fibers is greatly reduced in cultures made with B₁-deficient plasma. Using the Chi Square test and four degrees of freedom, the probability that this reduction is not significant is less than 0.01. Omitting classes O and IV, which contain the smallest number of cases, and using two degrees of freedom, P is still less than 0.01.

Thus it may be safely concluded that nerve fibers under these conditions "grow" less densely than in normal plasma.

Data on nerve fiber length in the same set of cultures is presented in table 2. Mean length of nerve fibers in B₁-deficient cultures is nearly 50% less than in control cultures, but because of the very large standard deviations obtained, the difference between means is not significant.

TABLE 2

Average length of nerve fibers observed in cultures of spinal ganglia after 2 to 3 days of incubation.

AVERAGE LENGTH ¹	NUMBER OF CULTURES OF GIVEN AVERAGE LENGTH IN		
	Normal plasma	Deficient plasma	Deficient plasma + B ₁
0	..	6	5
5	4	7	6
10	6	3	6
15	5	5	2
20	10	3	2
25	5	2	2
30	6	..	3
35	2	1	1
40	1	3	..
45	1	..	..
50	..	..	..
55	1	..	1 ³
60	..	..	..
65	..	..	..
70	..	1 ³	..
Total no. cultures	41	31	28
Mean length ¹	21.20	13.50	12.40
S.D. ¹	11.05	12.45	10.60
Mean length ²	0.34	0.21	0.20
S.D. ²	0.18	0.20	0.17

¹ Statistic expressed in ocular micrometer units.

² Statistic expressed in millimeters.

³ Not included in calculation of mean and standard deviation.

However, when the data are grouped into class intervals of ten ocular micrometer units each and the Chi Square test is applied, P is slightly above 0.01, so that the difference in distribution of nerve fiber lengths in the two populations of cultures is probably significant (table 3).

In measuring nerve fiber lengths no differentiation was made between those axons which ended free in the medium and those which were attached to spindle cells or macrophages. Such attachment must be considered in discussing axon elongation, because, when it occurs,

the bound axon is towed through the medium by the migrating cell (P. Weiss, '41), and for this reason the axon lengths recorded in table 2 are partially correlated with the density of non-nervous elements present in the same cultures.

Referring again to table 1, a statistically significant decrease in spindle cell density in vitamin deficient plasma was found both by analyzing the data as a whole and by analyzing only classes I and II. As for macrophages, the case is not equally clear. The probability that no significant difference in macrophage density existed between control and deficient cultures was between 0.05 and 0.10 when all the data were tested, and between 0.10 and 0.20 when class IV was omitted. Although these figures may indicate a possible effect on macrophage concentration under the conditions of the experiment, they are not statistically significant.

TABLE 3

Statistical analysis of nerve fiber length in cultures of spinal ganglia.

CLASS INTERVAL	NORMAL CONTROLS	DEFICIENT PLASMA	DEFICIENT PLASMA + B ₁	CHI SQUARE	
				Controls: deficient	Deficient: Deficient + B ₁
0-9	4	13	11	7.17	0.00
10-19	11	8	8	0.00	0.00
20-29	15	5	4	3.24	0.00
Over 30	11	5	5	1.02	0.00
Total	41	31	28	11.43	0.00

Not only did the deficient plasma affect cell density in the growth zones of the ganglia cultured in it, but it produced a marked effect on the appearance of the ganglia themselves. Thus, whereas living healthy ganglia cultured in normal plasma are translucent when examined microscopically by transmitted light, ganglia which have been cultured for a day or more in B₁-deficient plasma appear black and opaque under the same conditions, very much as if the medium had been toxic as suggested by McCarrison and Sankaran.

As a further test of the effects of deficient plasma, twelve lumbo-sacral plexus and the attached ganglia were dissected out of 14-day chick embryos. Instead of culturing these preparations in single paraffin rings, two rings were stamped on a piece of mica side by side in such a fashion that the ganglia could be cultured in one ring while the attached sciatic nerve was allowed to grow in the other, a method previously used in this laboratory (P. Weiss, unpublished). In two cases both rings were filled with normal plasma, and in two others

both were filled with deficient plasma. In four cases the ganglia were cultured in normal and the nerve in deficient plasma; in the remaining four cases the reserve relation was tested.

In normal plasma an abundant growth of spindle cells occurs within 24 hours from all parts of such a plexus preparation. Nerve fibers grow out from the ganglia and sometimes from the nerve as well, and occasional macrophages are found. When both nerve and ganglia lay in deficient plasma, macrophages were the only cells that migrated into the surrounding medium. In the mixed preparations, ganglia cultured in normal plasma behaved as usual, whereas the only cells that wandered out from the attached nerves were macrophages. In the opposite case where the ganglia were in deficient plasma and the nerve in normal plasma, nerve fibers grew from neither ganglia nor nerves, only macrophages appeared from the ganglia, and the growth of spindle cells from the nerve appeared to be markedly reduced. It was concluded that growth in normal plasma from nerves attached to ganglia lying in B₁-deficient plasma, as well as growth from nerves and ganglia cultured directly in deficient plasma is definitely impaired. However, whether this depression, or even suppression, of growth is due to the absence of thiamin or to the presence of toxic agents in the medium cannot be decided from these experiments. To test this point, the following series was undertaken.

Effects of addition of thiamin chloride to thiamin deficient plasma

If the inhibition of growth in deficient plasma is due directly to lack of vitamin B₁, then growth in deficient plasma to which thiamin has been added should approximate that in the control cultures, provided that spinal ganglion cells can utilize thiamin chloride which has not been previously phosphorylated. What actually happens when B₁ is added to deficient plasma may be seen from tables 1, 2, and 3.

No significant difference was found between deficient cultures to which thiamin had been added and those without it as to length or density of nerve fibers or density of macrophages. Thus the inhibition observed in the earlier series does not seem to be a primary result of lack of vitamin. However, there is a very strong probability that spindle cell growth under these conditions is improved by addition of B₁ (P lies between 0.05 and 0.01, even when class III is not considered). Even so, as may be seen by comparing the distribution of cultures among the five classes of spindle cell growth in table 1, thiamin per se does not restore growth to the level attained in normal plasma.

It should be noted that the method of measuring density which has been used is not as well adapted to spindle cell growth as it is to axon growth because individual nerve fibers can be easily seen in stained cultures whereas it is not possible to judge the volume of spindle cells present with great accuracy by inspection (Cunningham and Kirk, '42). The work reported here was designed mainly to study nerve growth, but in view of the suggestive results obtained with other cells, a re-examination of the problem using a more sensitive method of measurement for these elements would be desirable.

From the work reported above it seems evident that spindle cells are directly affected by lack of thiamin, since addition of that compound to a B₁-deficient medium improves their growth. For nerve fibers, on the other hand, the case is not equally clear, since the failure of thiamin to improve axon growth in B₁-deficient plasma cannot be regarded as conclusive until it has been proved that nerve fibers can utilize the non-phosphorylated vitamin in tissue culture, which fact has not yet been demonstrated beyond question.

However, as previously mentioned, there are other factors involved in growth in B₁-deficient plasma besides the lack of thiamin. Among these are (1) accumulation of possibly toxic intermediary products of carbohydrate metabolism in deficient blood, and (2) changes in the physical consistency of deficient plasma as compared with the normal condition.

Effects of intermediary metabolites on growth of spinal ganglia

To test the first of the above factors, cultures of 10-day chick ganglia were made in normal plasma to serve as controls and in plasma to which 50 mg. % of lactic acid or aceto-acetic acid had been added, and cultures of 15-day chick ganglia were made in normal plasma with and without 50 mg. % pyruvic acid added. As cocarboxylase is necessary for the breakdown of pyruvic acid in carbohydrate metabolism, the accumulation of pyruvic acid mentioned in the introduction to this paper is only to be expected in beri-beri. Kinnersley and Peters ('29) have demonstrated that lactic acid also accumulates in B₁-deficient pigeons, and aceto-acetic acid is believed by Wilkins, Taylor, and S. Weiss ('37) to be one of the bisulfite binding substances occurring in human B₁-deficiency. In all cases the chemicals were added to modified Tyrode's solution and neutralized before addition to the plasma, as the pH of deficient plasma was found to be normal both by McCarrison and Sankaran and by the author.

Although the amounts of intermediary metabolites added exceeded the b.b.s. level in severe beri-beri, in no case was density of axons or fibroblasts reduced to the level characteristic of deficient cultures (table 4). Concomitantly, there was no significant reduction of nerve fiber length in the metabolite treated cultures. Inasmuch as addition of these intermediary metabolites to normal plasma does not duplicate the effects of vitamin deficient plasma on spinal ganglia, it may be concluded that the presence of the tested compounds as such is not responsible for the inhibitory action observed.³ Further implications and qualifications of this work will be discussed later.

TABLE 4

Average density of cells in cultures of spinal ganglia expressed in arbitrary class units.

	NERVE FIBERS	SPINDEL CELLS	MACROPHAGES
Normal controls	2.9	2.8	2.7
Lactic acid	2.8	2.5	3.2
Aceto-acetic acid	2.2	2.8	3.4
Pyruvic acid	2.7	2.2	2.0
B ₁ deficient plasma ¹	1.1	1.0	2.2

¹ Average density in deficient plasma was calculated from data in table 1.

Not only did the plasma containing the tested metabolites fail to affect cell density to the same degree as deficient plasma does, but it failed to duplicate the appearance of vitamin deficient cultures; this leads to a consideration of the next factor involved in growth in deficient plasma.

Effects of consistency of the medium on growth of spinal ganglia

As P. Weiss ('34) has shown, the type of nerve growth which occurs in tissue culture is largely determined by the physical organization of the substratum. Indications of the type of organization present in plasma clots may be obtained from (1) the length and direction of axon growth, and (2) the shape of spindle cells.

As noted by W. and M. Lewis ('12), Mihálik ('32), and W. Lewis ('39), nerve fibers grown in a liquid medium are straight, whereas those grown in more solid plasma clots assume a wavy course. As Weiss has demonstrated, the course of a nerve fiber which is not under

³ Acetaldehyde, believed by Shindo ('37) to occur in beri-beri, also failed to duplicate the effects of avitaminotic plasma in tissue culture. (Nerve fiber density, 2.5; spindle cells, 2.0; macrophages, 3.0.) These results are not included in table 4 as there is now good reason to believe Shindo's findings were in error (Barron, '41).

tension is normally wavy, while straight fibers are found only when some sort of tension is being exerted on them, such as the pull of a migrating cell to which the fiber may become attached or of surface tension acting at the phase boundaries of partially liquid cultures. Where straight fibers are found which are not being towed by non-nervous cells, as they are in many cultures made with B₁-deficient plasma, they therefore indicate liquidity of the medium. In most of the ganglia cultured in deficient plasma from which nerve growth occurred, the axons were either all straight and of the liquid medium type, or were of the liquid-medium type in some areas around the ganglion, and of the wavy plasma type in others. Figure 1 A is a photograph of an 11-day chick ganglion cultured in deficient plasma which illustrates liquid-type nerve growth, while figure 1 B is a photograph of axons cultured in normal plasma which illustrates plasma-type nerve growth for comparison.

Furthermore, under favorable conditions axons growing in liquid medium attain a greater total length than do those in plasma (Mihálik, '32), probably because they are drawn out passively by surface tension as well as pushed out as active pseudopodia from the neuron (Weiss, '34). In a few cases, such long axons were found in deficient plasma preparations (table 2).

Physical changes in deficient plasma were observed by McCarrison and Sankaran, who noted a change in its color and consistency. The deficient plasma used in the experiments described here formed very loose clots the interior of which was sometimes found to be liquid on dissection.

Early regression of nerve fibers which was observed by McCarrison and Sankaran and occasionally by the author, has frequently been observed in liquid-medium cultures when, for some reason, axons lose their hold on the interface and float free in the culture. Failure of axons to emerge at all from ganglia, as occurred in six of the thirty-four deficient cultures, is also characteristic of liquid medium cultures under unfavorable conditions. However, it has, on the whole, been the experience in this laboratory that quantity of axon growth from ganglia in liquid tissue culture media compares favorably with that in plasma clots, in spite of the different growth form assumed, so that the change in physical consistency of the deficient plasma clots would not in itself account for the observed suppression of nerve fibers.

Liquidity of the medium may affect the ratio of spindle cell to macrophage frequencies. Thus, when spinal ganglia are grown in liquid

media, the emerging sheath cells frequently transform into macrophages. The effects of this change were particularly striking in the living cultures where occasionally, after 2 days' incubation, no typical spindle cells remained and the ganglion would appear completely surrounded by macrophages. Figure 1 C shows such a ganglion incubated in deficient plasma, while figure 1 D is a photograph of a living culture in normal plasma which illustrates the normal growth pattern of fibroblasts and sheath cells.

This fact must be borne in mind when interpreting the observations on spindle cell and macrophage density in vitamin deficient cultures as compared with controls. Thus some of the reduction in spindle cell density which has been demonstrated to occur in deficient plasma may be accounted for by transformation of these cells into macrophages. Likewise, the reduction in numbers of original macrophages emerging from ganglia in deficient plasma may be greater than shown in the tables. However, regardless of the modulation that takes place, it is apparent that the sum of the non-nervous elements in B₁-deficient cultures is less than that in the normal controls.

Furthermore, addition of thiamin to deficient plasma apparently does not affect its physical consistency, yet to a large degree restores spindle cell growth. Therefore, as in the case of nerve fibers also, the physical constitution of the deficient plasma cannot be regarded as the main cause of the inhibition of the non-nervous elements.

Growth in artificial B₁-deficient media

Inasmuch as the physical conditions in cultures made in B₁-deficient plasma can be shown to affect the type of growth resulting, such cultures cannot be accepted as completely valid tests of the effects of lack of thiamin per se on growing axons. Consequently, cultures were made in two media which presumably contained no thiamin, in one of which the physical constitution of normal plasma was preserved, while the other was completely liquid. These were, respectively, (1) normal plasma which had been washed with sterile 0.1% sodium sulfite, shown by Williams ('35) to split thiamin into two inactive fractions, and (2) pure Tyrode's solution.

In four sulfite washed plasma clots, nerve growth was normal both as to quantity and quality, and in four plasma clots on which 0.1% sodium sulfite was allowed to remain during the period of cultivation, some outgrowth of nerve fibers was observed, although the cultures were by no means typical, due, no doubt, to the toxic action of the

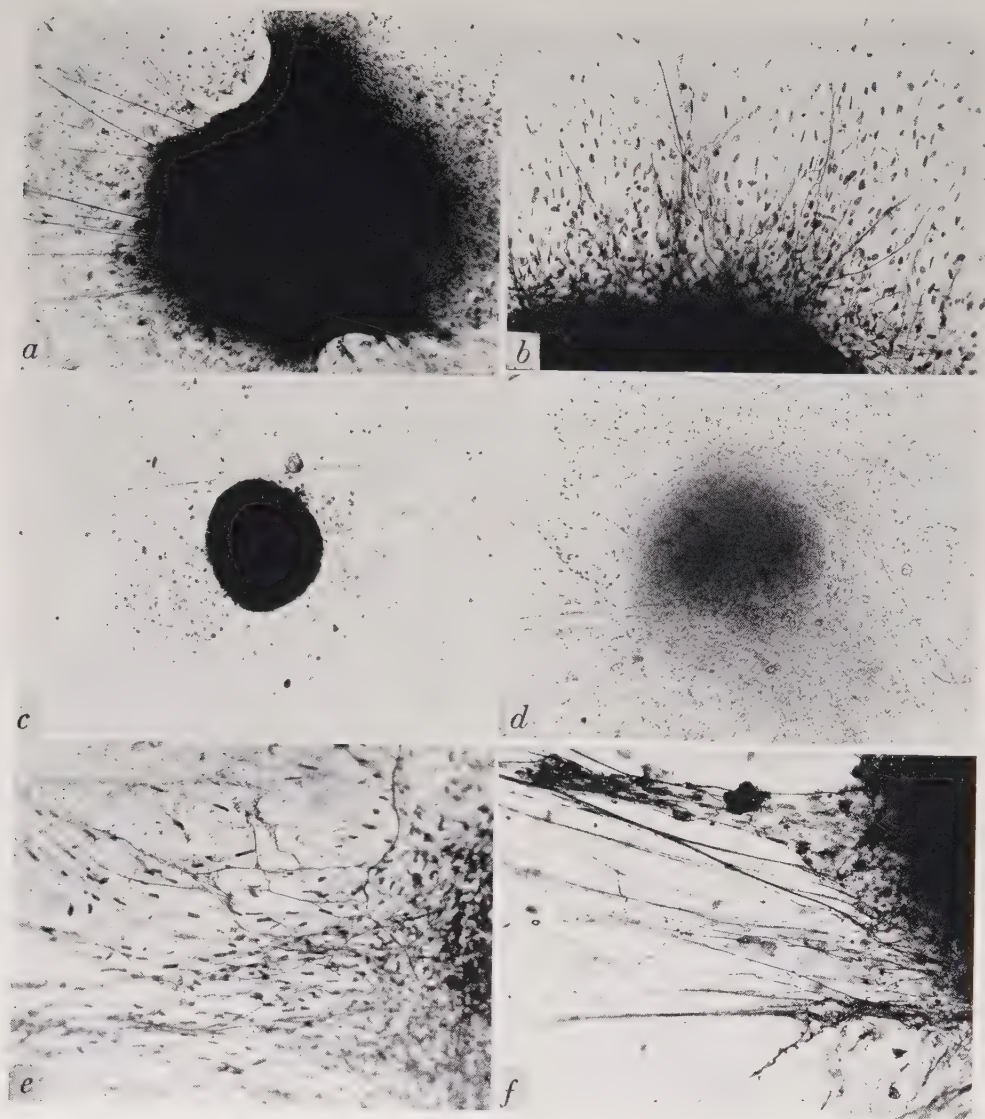


Figure 1

- a. Ganglion cultured in B_1 deficient plasma showing liquid medium type nerve growth on left side and plasma type spindle cell growth on right. Bodian's protargol stain. $\times 53$.
- b. Ganglion cultured in normal plasma showing plasma type axon growth. Bodian's protargol stain. $\times 95$.
- c. Living, unstained ganglion cultured in B_1 -deficient plasma. Note the opaque appearance of the ganglion, the abundance of macrophages, and the absence of other cells. $\times 32$.
- d. Living, unstained ganglion cultured in normal chicken plasma. Compare with figure 1 C. $\times 32$.
- e. Portion of a ganglion cultured 3 days in normal chicken plasma plus 0.1% sodium sulfite. Note plasma type axons present. Bodian's protargol stain. $\times 165$.
- f. Axon fibers emerging from a ganglion cultured 3 days in pure Tyrode's solution. Bodian's protargol stain. $\times 165$.

sulfite. One of these cultures is shown in figure 1 E. Chemical analysis of the sulfite treated plasma would be necessary to confirm the absence of thiamin, but the results as they stand suggest strongly that the presence of thiamin in the culture medium is not necessary for axon outgrowth to take place.

The same conclusion was reached after a study of the behavior of ganglia in Tyrode's solution, for, if ganglia are made to adhere to the cover slip before liquid medium is added, dense outgrowth of nerve fibers can sometimes be obtained even in purely inorganic media (P. Weiss, unpublished observations). Figure 1 F shows such a ganglion after staining by Bodian's method. The axons present should be noted.

DISCUSSION

To summarize the data presented here, significant inhibition of axon and spindle cell, but not of macrophage density has been demonstrated in tissue cultures growing in plasma from B₁-deficient chickens. This inhibition is partially removed by addition of thamin chloride to the medium in the case of spindle cells but not of nerve fibers. Addition of various intermediary metabolites which may accumulate in the intact animal with beri-beri does not reproduce the picture seen in tissue cultures in deficient plasma. Evidence has been presented that the type of growth obtained in this medium is affected at least in part by its greater liquidity as compared to normal plasma, and, finally, normal axon outgrowth has been obtained in media containing little or no vitamin B₁.

While a definite inhibitory effect of vitamin deficient plasma on axon growth may be regarded as established by this work, the causes for the inhibition are not yet obvious. Three possibilities which can, however, be considered are (1) that the inhibition is caused by lack of thiamin, (2) that it is caused by unfavorable physical organization of the plasma clot, and (3) that it is caused by toxic substances present in the plasma.

Most of the available evidence fails to support the first hypothesis, for, although no one has shown that non-phosphorylated thiamin can be utilized by nerve cells and the failure of thiamin to restore axon growth in B₁-deficient plasma does not necessarily mean that lack of thiamin is responsible for that lack of growth, still the fact remains that axons will elongate in sulfite washed plasma and in pure salt solutions, neither of which media should contain thiamin other than that which might diffuse out of the ganglion itself. It is possible, of course, that the ganglia tested may have contained enough thiamin to suffice for axon

elongation during the short periods of time for which they were studied. The fact that addition of thiamin to deficient plasma produces marked effects on spindle cell growth might indicate either that spindle cells contain less stored thiamin than neuroblasts or that their thiamin requirements are higher.

The failure of thiamin to affect axon growth in deficient plasma is of interest in view of previous work on the effects of excess vitamin. Although Hengstmann ('39) believes that thiamin stimulates growth of connective tissue cells cultured in normal plasma, and Vollmar ('39) claims to have similarly stimulated nerve growth *in vitro*, their data have been presented in such an inadequate fashion that it is not possible to judge the validity of their findings, which have not been supported by statistical analysis of thiamin treated cultures in this laboratory (Burt, '43). Likewise, although Tangari ('40) reports an increased nerve regeneration rate in rats to which thiamin was administered, Fischer ('43) is unable to affect either the rate of regeneration of crushed rat sciatics or the rate of atrophy of denervated rat gastrocnemii by administration of pure thiamin chloride or of yeast. Thus it seems doubtful that B_1 stimulates nerve growth *in vivo*. In line with these findings is the fact that decarboxylation of pyruvic acid by yeast protein is not increased by addition of thiamin above the optimum level, but is proportional to the amount of protein present rather than to the amount of vitamin (Weil-Malherbe, '39).

The observations on the physical appearance of cultures in B_1 -deficient plasma indicate clearly that the ganglia cultured in it were in a more liquid milieu than those in normal plasma. This factor has been shown to affect the modulation of spindle cells and consequently the concentration of macrophages, but the extent of its effect on axon growth cannot be decided with certainty from the work at hand because the growth of axons in liquid media depends to a large extent on the way cultures are prepared and on the degree of contact between the ganglia and the surface of the cover slip. As no special precautions were taken in explanting the cultures grown in deficient plasma such as are taken as a matter of routine when they are grown in salt solutions, it is possible that some of the inhibition of fiber growth observed may have been due to failure of the axons to find an interface along which to grow. However, the presence of transforming sheath cells in the deficient cultures indicates that there was fairly good adherence of the ganglia to the mica in most cases; also, while failure of the ganglia to stick to the substrate might account for failure of the axons to emerge

in some cases, it would not explain why those that did were characteristically shorter than those in normal plasma. On the contrary, if degree of liquidity were the only difference between the two types of plasma, one would expect a greater total length in the deficient medium. Nor does physical organization as such explain why, in the lumbo-sacral plexus preparations described on page 210, cell growth was inhibited from the nerves which were embedded in normal plasma and attached to ganglia cultured in deficient plasma, or why the spinal ganglia cultured in B₁-deficient plasma assumed an opaque, unhealthy appearance after 1 day, while ganglia in normal plasma or in salt solution will remain translucent for several days irrespective of whether or not they are attached to the substratum.

Although none of the intermediary carbohydrate metabolites tested in this work were shown to be toxic for nerve growth in the concentrations in which they occur in beri-beri, this does not eliminate the possibility that toxic agents may be present in blood from B₁-deficient animals. For one thing, by no means all the possible intermediary metabolites have been tested. Thus methyl glyoxal, which has been found by Geiger and Rosenberg ('33) in children suffering from B₁-deficiency and which is known to be toxic when injected into intact animals, although it does not produce the symptoms of beri-beri (cf. Haynes and S. Weiss, '40), was unfortunately not available at the time this work was done, and it is but one of many compounds which could conceivably occur in vitamin deficiency and whose effect on tissue cultures is not known.

Furthermore, metabolites which are non-toxic themselves, such as pyruvic acid, may undergo complicated reactions in the blood which, after a sufficient length of time, could produce highly injurious compounds (Lu, '39 b). It is quite possible that this might occur in vivo in the course of a vitamin deficiency lasting several weeks yet not show up in a short time tissue culture experiment. Also, it must be borne in mind that long continued thiamin deficiency produces pathological changes in nearly all the organ systems of the animal affected (Eddy and Dalldorf, '41), so that blood from a deficient animal might contain toxic agents arising from other causes than a disordered carbohydrate metabolism. A thorough chemical analysis of plasma from B₁-deficient chickens would seem to be a prerequisite for further work along this line and would be advisable because, although the reason for inhibition of nerve growth in deficient plasma cannot yet be assigned with certainty, the hypothesis that a toxic agent is responsible looks the most promising.

In conclusion, it should be emphasized that the problems of nerve growth in tissue culture and of metabolism in vitamin deficiencies are very complex and that broad generalizations from the simple experiments reported here would not be justified. This is particularly true for the question of what causes nerve lesions in chronic B₁-deficiency. However, the phase of axon growth studied in this paper is the one which is significant in the first stages of nerve regeneration, and although later phases of regeneration must be studied separately in long term experiments, any information which can be gathered about this stage of nerve growth will aid in elucidating suture line phenomena.

SUMMARY

1. When spinal ganglia of 9- to 15-day embryonic chicks are grown in plasma from vitamin B₁ deficient chickens, the length and density of nerve fibers and density of spindle cells is significantly reduced as compared to cultures grown in normal plasma, but macrophage density is not affected to a statistically significant degree.

2. Addition of 4 mg. % thiamin chloride to such deficient plasma does not affect nerve fiber length or density or macrophage density, but spindle cell density is significantly increased.

3. Abundant growth from all parts of embryonic chick lumbo-sacral plexus occurs in tissue culture. When either the ganglia or nerve or both lie in deficient plasma, only macrophages appear. When the ganglia are in deficient and the nerve in normal plasma, spindle cell outgrowth from the nerve seems to be reduced.

4. Addition of 50 mg. % pyruvic acid, lactic acid, acetaldehyde, or aceto-acetic acid to cultures of spinal ganglia does not reduce growth density to the levels observed in deficient plasma, nor are the physical aspects of deficient cultures reproduced.

5. Evidence is presented that differences in axon growth form in deficient cultures as compared to those in normal plasma are due to greater liquidity of deficient plasma. The same factor brings about a modulation of spindle cells into macrophages in deficient cultures.

6. Outgrowth of axons into pure salt solutions containing no thiamin has been observed. Typical nerve growth has also been seen in plasma clots washed with sodium sulfite which splits thiamin into two inactive components, and growth of an abnormal variety has been observed in clots on which the sulfite was allowed to remain during incubation. Thus the presence of thiamin in the culture medium may be unnecessary for the initial stages of axon growth.

7. The reason for the inhibition of axon growth in deficient plasma cannot be assigned with certainty on the basis of the work reported here because

(a) thiamin added to B₁-deficient plasma does not improve axon growth.

(b) nerve growth is not suppressed in the presence of those intermediary carbohydrate metabolites which have been tested.

(c) the demonstrated physical differences between normal and B₁-deficient plasma are not sufficient to account for the observed reduction in nerve fiber density.

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